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The Impact of Diabetes Mellitus on the Association of Endothelial Nitric Oxide Synthase Gene Polymorphisms (4a/4b, G894T, and T786C) with Clopidogrel Resistance in Coronary Artery Disease Patients Undergoing Percutaneous Coronary Interventions

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ABSTRACT

Background: Compared with the general population, patients with diabetes mellitus (DM) usually show higher platelet reactivity, which increases their risk of atherothrombotic events, such as acute coronary syndrome, during the percutaneous coronary intervention (PCI). Endothelial nitric oxide synthase (eNOS) gene polymorphisms have been associated with many diseases, but their role as risk factors of coronary artery disease (CAD) in DM is yet unknown. Although acetylsalicylic acid and clopidogrel are prescribed individually or in combination for high-cardiovascular risk patients with overall improvements in outcomes, DM patients are still at greater risk of recurrent ischemic events.

Objective: This study was conducted to determine the effect of DM on the association of e-NOS polymorphisms with clopidogrel resistance in patients with CAD undergoing PCI.

Methods: Three-hundred twenty-four of CAD patients were recruited in this case-control study. All patients were subjected to platelets function test within at least two hours before PCI techniques to determine the clopidogrel resistance first then classified accordingly to clopidogrel resistance group (case group n=111) and non-clopidogrel resistance group (control group n=213). Each group was subdivided into a diabetic patient's group and non-diabetic patients' group. Blood samples were taken for genotyping analysis and phenotyping determination by measuring the level of nitric oxide in the plasma via colorimetric method. The genotype analysis was carried using allele-specific polymerase chain reaction based on the banding pattern on gel electrophoresis(GEP) to evaluate whether genetic e-NOS polymorphisms (**T786C**, exon 7 **G894T** and intron 4 (**4a4b**) affect NO formation in both groups. Odds ratios for these polymorphisms were then estimated for the clopidogrel and non-clopidogrel resistance groups.

Results: Approximately 34.25% of the study population showed clopidogrel resistance. No significant difference in serum NO levels between the case and control groups (26.1 ± 1.52 µM vs. 25.41 ± 1.18 µM; P = 0.727) was observed.

The effect of diabetes on clopidogrel resistance was significant in the case group (20.06 **±** 1.82 µM) in diabetes patients when compared with 26.52 **±** 2.40 µM in non- diabetes patients (P = 0.03), but within a control group, the effect diabetes on clopidogrel resistance was not significant (P = 0.07), while the effect of diabetes was not significant in diabetes patient (P = 0.09) and non-diabetes (P = 0.4) when the comparison between case and control. The study found that e-NOS intron 4 **(4a4b)** polymorphisms genotypes were significantly associated in the diabetic group as compared with non –diabetic group in patients' resistant to clopidogrel [**ab** genotype: OR = 2.4 (1.1–5.69), P=0.02; **aa** genotype: OR = 2.8 (0.89–8.7), P=0.03], while there was a negative association between diabetic group and non–diabetic group in patients' sensitive to clopidogrel. However, e-NOS polymorphisms of the **T786C** and exon 7 **G894T** genotypes showed no association between diabetic and non-diabetic patients of both resistant or sensitive patients except mutant homozygous **CC** of **T786C** showed a significant association between diabetic and non-diabetic patients in case and control groups (P = 0.02, 0.04) respectively.

Conclusions: Our study indicates that e-NOS gene polymorphisms, specifically the **4a** allele (**4a/ab**) and mutant homozygous **CC** (**T786C**), maybe determinants of clopidogrel resistance in diabetic CAD patients.

Keywords: Diabetes mellitus, coronary artery disease, clopidogrel resistance, endothelial nitric oxide synthase gene polymorphisms, and percutaneous coronary intervention.

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INTRODUCTION

Diabetes mellitus is a disease that currently affects over 150 million individuals globally [1]. DM is usually related to enhanced atherosclerosis and could result in premature CAD, which is the leading cause of mortality in developed nations [2]. Patients with DM have a 2-4-fold increased risk of developing CAD. Frequent ischemic events are more common in patients with DM than in non-DM patients [3]. Platelet dysfunction contributes to the high risk of atherothrombotic complications in the diabetic

population [4]. Altered platelet function manifests as hypersensitivity to aggregants observed in in vitro studies. The problems associated with DM include albuminuria, diabetic neuropathy, renal failure, diabetic retinopathy, cardiac disease, hypertension, obesity, and other vascular diseases [5]. DM and CAD are pathological conditions wherein reduced nitric oxide (NO) may be present. NO can regulate inflammation, coagulation, and vascular tone. Reduced NO action may be attributed to impair NO production [6]. Several e-NOS polymorphisms have been detected in numerous populations. A 27-base pair (bp) repeat polymorphism in intron 4 of the (e-NOS **4a/4b**) has been described. The single nucleotide polymorphism (SNP) **T786C**, in which thymine (**T**) is replaced with cytosine (**C**), has been identified in the 5'-flanking region of e-NOS at a locus 786 bp upstream of the first exon. An additional common variant of e-NOS resulting in a **G**-to-**T** conversion at nucleotide position 894 (**G894T**) accompanied by a change in amino acid 298 (Glu298asp) has been detected [7] [8].

Dual antiplatelet treatment using clopidogrel and acetylsalicylic acid (ASA) is the usual protocol for the secondary prevention of cardiovascular events after the implantation of coronary stents in stable CAD patients [9]. However, variable intra- and inter-individual responses to clopidogrel, such as low responsiveness or resistance, represent a significant clinical problem [10]. Moreover, several drug interactions have been recorded to affect the inhibition of platelet function mediated by clopidogrel. For example, a significantly increased cardiovascular risk accompanied by incompetent suppression of platelet activity has been reported following ASA and clopidogrel therapy [10]. Thus far, no study has yet been carried out to elucidate the role of DM on the association between e-NOS polymorphisms and clopidogrel resistance in Iraqi patients with DM. The present case-control study was performed to determine the effect of DM on the association of e-NOS polymorphisms (**4a/4b, G894T**, and **T786C**) with the development of clopidogrel resistance in CAD patients undergoing PCI.

MRTHODS

Study design

This work represents a case-control study conducted from October 2018 to February 2019 at the Annajaf Heart Surgery and Catheterization Center in Annajaf governorate of Iraq. The participants included 370 male and female patients of Arabic race and Iraqi nationality aged between 35 and 70 years. All participants were angiographically diagnosed with CAD confirmed by at least two cardiologists according to the center's policy for patients presenting with ≤70% stenosis affecting at least one coronary vessel. Of the 370 patients initially recruited, 46 were excluded because they did not meet inclusion criteria; thus, 324 patients were finally included in this work. All patients suffering CAD were first subjected to a platelet reactivity test within at least 2 hours before PCI to determine the percentage of clopidogrel resistance and then classified into the clopidogrel resistance group (case group) and the non-clopidogrel resistance group (control group)[11]. NO levels were then determined by the colorimetric method.

Patients

The case group included 111 patients (31.2% males, 41.9% females), while the control group included 213 patients (68.8% males, 58.1% females). The sample size was calculated using a website that calculates the sample size for case-control studies. All participants were interviewed to collect data regarding family history of CAD, diabetes, hypertension, body mass index (BMI), and smoking status using a questionnaire. The data of this study included baseline and angiographic characteristics; interventional results were obtained from the Annajaf Heart Surgery and Catheterization Center information system. A family history of CAD was considered positive when at least one first-degree relative was identified with CAD by the age of 55 years for males or 65 years for females [12]. All patients were verbally informed about the procedure and purpose of the research and provided written informed consent before participating in the study.

Exclusion criteria

- History of bleeding diathesis
- Platelet count 100,000/mm; hematocrit, 30%
- Age > 70 years
- Renal or hepatic impairment
- Pregnancy
- Emergency procedure

Collection and preparation of blood samples

Approximately 3 ml of venous blood was collected from each patient into EDTA tubes for serum NO level measurements and platelet function tests. The remaining blood was stored at −70 °C for extraction of DNA and identification of the various genetic patterns of endothelial nitric oxide synthase (e-NOS) polymorphisms by polymerase chain reaction (PCR). Samples were centrifuged for 15 min at 1000×*g* and 2–8 °C. Supernatant plasma was collected in plain tubes and frozen at −20 °C for NO assay.

Ethical considerations: The study protocol was approved by the ethics committee of the College of Medicine, University of Kufa. All of the procedures were explained to all patients, and informed consent was obtained.

Genotyping analysis

DNA was extracted by using Column – pure Blood Genomic DNA Mini Kit (Extraction Kit) (Abm /Canada).

The primer orders to identify the 4a/4b polymorphism were

5'- ATCAGGCCCTATGGTAGTGC -3' and 5'-TCTCTTAGTGCT-

-GTGCTCAC-3'. The amplification of DNA for 40 cycles of denaturing at 94˚C /280 sec, then annealing at 62˚C for 30 sec and extension at 72˚C for 60 sec. By GEP, the PCR products were visualized [13].

The forward and reverse primers employed to identify the G894T polymorphism were 5'-CATGAGGCTCAGCCCCAGAAC-3' and 5'-AGTCAATCCCTTTGGTGCTCAC-3', respectively. PCR was carried out in 40 cycles of 94 °C for 280 s, 62 °C for 30 s, and 72 °C for 60 s. The PCR products were digested with the restriction endonuclease MboI (New England Biolab, UK) at 37 °C for 16 h and examined by GEP [14].

The primers used to detect the e-NOS polymorphism T786C were 5'-CAGATGCCCAGCTAGTGG-3' and 5'-GGACCTC-TAGGGTCATGC-3'. DNA was amplified via 40 cycles of denaturation at 94 °C for 280 s, annealing at 62 °C for 30 s, and extension at 72 °C for 60 s. The PCR products were examined by GEP after incubation with the restriction endonuclease MspI (New England Biolab) at 37 °C for 16 h [15].

Estimation of plasma nitric oxide

The determination of NO in serum samples was performed indirectly via nitrite assay using the colorimetric method. Nitrite and nitrate are important chemical components in nearly all living organisms. In vivo, nitrite and nitrate are produced by the NO oxidation pathway. Nitrite can react with a chromogenic agent to produce a light-red azo-compound. Nitrite contents can be calculated indirectly by measuring the OD value at 550 nm [16].

Clopidogrel loading:

In the appointment room, the patients were recommended to take 75 mg of clopidogrel (Acino) daily for 8 days (total dose, 600 mg) before PCR day. We certify that all patients finished their required doses.

Platelets Function Test

Blood samples (5 ml) to test for platelet aggregation were placed in tubes for 1 h and centrifuged at 3000 rpm for 10 min at room temperature for plasma and serum separation. The platelet function test was done using the Multiplate analyzer® (GmbH, Munich, Germany, Verum Diagnostica). Samples of blood were evaluated during a period of half to three hours after the collection of blood. The analysis was specified by the AUC (area under the aggregation curve= Area under the Curve) in U (units). In our study, the clopidogrel resistance cut-off point is ≥50 U [17].

Statistical analysis

All of the data in this study were analyzed using SPSS version 25 and presented using descriptive statistics, such as means, standard deviations, numbers, and percentages. Mean ± standard error (SE) was used to express age and BMI. Categorical data and associations between categorical variables were analyzed using the chi-squared test. Numerical data and comparisons between the case and control groups were analyzed using independent *t*-tests. Odds ratios at the 95% confidence level were used. P values less than 0.05 were regarded as significant.

RESULTS

The clinical features and demographic data of the 324 CAD patients, including 56 DM patients in the CR group and 119 DM patients in the non-CR group, are detailed in Table (1). No significant differences in age, gender, hypertension, smoking status, CAD maturity, family history, DM, BMI, and types of medications taken (e.g., ACEIs, ARBs, CCBs, β-blockers, diuretics, and atorvastatin) were observed between these groups.

Table 1. demographic and angiographic data of the patient with CAD(n=324)

Variables		Case n (111)	Control n (213)	P
Age/years	Mean ±SD	56.03±9.15	57.63±7.94	0.103
Gender	Female n (%)	39 (41.9)	54 (58.1)	0.065
	Male n (%)	72 (31.2)	159 (68.8)	
Hypertension	Yes	74 (66.7)	144 (67.7)	0.864
Smoking	Smoker n (%)	35 (31.5)	64 (30)	0.827
	Ex-smoker n (%)	22 (19.8)	38 (17.8)	
	Non-smoker n (%)	54 (48.6)	111 (52.1)	
Diabetes	Yes	56 (50.5)	119 (55.9)	0.353
CAD maturity	Yes	57 (51.4)	101 (47.4)	0.501
	No	54 (48.6)	112 (52.6)	
Family history	Yes	38 (34.2)	82 (38.5)	0.451
BMI	Mean ±SD	29.25±4.93	28.42±4.59	0.129
Type of medication used	ACEIs n (%)	25 (22.5)	43 (20.2)	0.624
	ARBs n (%)	38 (34.2)	54 (25.4)	0.092
	CCBs n (%)	22 (19.8)	37 (17.4)	0.588
	β-blockers n (%)	69 (62.2)	134 (62.9)	0.895
	Diuretics n (%)	30 (27)	50 (23.5)	0.482
Atorvastatin	20mg n (%)	37 (42)	94 (48)	0.875
	40mg n (%)	51 (58)	102 (52)	0.393

The effect of DM on clopidogrel resistance was significant within a case group as 20.06 ± 1.82 μM in DM patients when compared with 26.52 ± 2.40 μM in non-DM patients (P = 0.03), but within a control group the effect DM on clopidogrel resistance was not significant as 24.99 ± 1.81 μM in DM patients when compared with 29.56 ± 2.38 μM in non-DM patients (P = 0.07; Table (2)).

But the effect of DM on clopidogrel resistance was not significant in DM patients when compared between case group 20.06±1.82 uM and control group 24.99 ± 1.81uM(P=0.09), and the effect of DM on clopidogrel resistance was not significant in non-DM patients when compared between case group 26.52 ± 2.40 uM and control group 29.56 ± 2.38 uM (P=0.4), Table (2).

Table 2. Association between the diabetes mellitus and clopidogrel resistance

DM	Case n (111)	Control n (213)	P
	Nitric oxide level uM	Nitric oxide level uM	
Yes	± 1.82 20.06	24.99 ± 1.81	0.09
No	26.52 ± 2.40	29.56 ± 2.38	0.40
P-value	0.03	0.07	

This study investigated three e-NOS polymorphisms, namely, 4a/4b, G894T, and T786C. The first SNP studied is 4a/4b. The effect of DM on the **ab** genotype distribution was significant in DM versus non-DM patients in the case group [OR = 2.4 (1.1–5.69); P = 0.02]. The effect of DM on the **ab** genotype distribution was not significant in DM versus non-DM patients in the control group [OR = 1.32 (0.74–2.35); P = 0.16; Table (3)].

Similarly, the effect of DM on the **aa** genotype distribution was significant in DM versus non-DM patients in the case group [OR = 2.8 (0.89–8.7); P = 0.03]. The effect of DM on the **aa** genotype distribution was not significant in DM versus non-DM patients in the control group [OR = 0.88 (0.32–2.45); P = 0.40; Table (3)].

The second SNP analyzed in this study is G894T. The effect of DM on the **GT** genotype distribution not significant in DM versus non-DM patients in the case [OR = 0.72 (0.31–1.69); P = 0.23] and control [OR = 1.25 (0.70–2.25); P = 0.22] groups [Table (3)]. Moreover, the effect of DM on the **TT** genotype distribution was not significant between the DM versus non-DM patients in the case [OR = 0.63 (0.1–4.0); P = 0.31] and control [OR = 0.95 (0.34–2.67); P = 0.46] groups [Table (3)].

Last SNP analyzed is this study is T786C. The effect of DM on the **TC** genotype distribution was not significant between DM and non-DM patients in the case [OR= 1.72 (0.74–3.99); P = 0.09] and control [OR= 1.41 (0.78–2.55); P = 0.12] groups. By contrast, the effect of DM on the **CC** genotype distribution was significant in DM versus non-DM patients in the case [OR = 9.07 (1.05–78.25); P = 0.02] and control [OR = 3 (0.86–10.45); P = 0.04] groups [Table (3)].

Table 3. The effect of Diabetes Mellitus on the association between e-NOS Polymorphisms (4a/4b G894T, and T786C) and clopidogrel resistance

Genotype	Resistant		OR Cl (%95)	P	Non-Resistant		OR Cl (%95)	P
	DM n=54	Non-DM n=57			DM n=94	Non-DM n=119		
4a/4b								
bb	22	37	1	1	48	67	1	
ab	20	14	2.4(1.1-5.69)	0.02	39	41	1.32(0.74-2.35)	0.16
aa	10	6	2.8(0.89-8.7)	0.03	7	11	0.88(0.32-2.45)	0.40
G894T								
GG	39	37	1	1	52	71	1	
GT	13	17	0.72(0.31-1.69)	0.23	35	38	1.25(0.70-2.25)	0.22
TT	2	3	0.63(0.1-4.0)	0.31	7	10	0.95(0.34-2.67)	0.46
T786C								
TT	27	35	1		54	81	1	
TC	20	15	1.72(0.74-3.99)	0.09	32	34	1.41(0.78-2.55)	0.12
CC	7	1	9.07(1.05-78.25)	0.02	8	4	3(0.86-10.45)	0.04

DISCUSSIOM

Polymorphisms of e-NOS are involved in several diseases, but their roles as risk factors for CAD in DM are mostly unknown. Previous research has not revealed an association between DM and the association e-NOS polymorphisms with clopidogrel resistance. To the best of our knowledge, this work is the first to examine the effect of DM on the association of e-NOS polymorphisms with clopidogrel resistance in patients with CAD undergoing PCI.

In the current study, all traditional risk factors, such as hypertension, diabetes, obesity, CAD maturity, and smoking, as well as age, gender, and medication, were taken into consideration. No association between these factors and clopidogrel resistance was found, consistent with many studies and inconsistent with others. DM was specifically found to have *no* relationship with clopidogrel resistance, which agrees with [18] but contrasts [19] and [20]. The diversity of results in these studies may be attributed to numerous factors, such as differences in selection criteria and population sizes, and the effects of factors such as race, age, and environmental and geographical differences. Moreover, differences may be correlated with the type and severity of disease and the subgrouping of CAD patients.

An interesting finding in the current study is that the rate of clopidogrel resistance in our Iraqi sample is 34.25%, which is higher than those found in other studies done in Iraq (18.3%, *n* = 115) [11], Jordan (32%, *n* = 270) [21], Iran (24.76%, *n* = 105) [22], and Turkey (19.8%, *n* = 207) [23]. However, the rate of clopidogrel resistance found this study was lower than those found in studies in Saudi Arabia (66%, *n* = 304) [24], Spain (44%, *n* = 48) [25], and Brazil (38.5%, *n* = 205) [26]. The global incidence of clopidogrel resistance is 5%–44% [27].

This study did not find any evidence of a marked association between the case and control groups in DM and non-DM patients except within the case group between DM and non-DM patients (P = 0.03). DM has been identified as a risk factor for cardiovascular diseases correlated with endothelial dysfunction by decreasing NO generation in blood vessels [28] [29]. A possible explanation for this result may be that hyperglycemia, through the production of ROS, could directly affect endothelial progenitor cells (EPCs) [30]. Ambasta *et al.* demonstrated that high glucose reduces the function, survival, and proliferation of cultured EPCs and decreases NO production and MMP-9 activity concomitantly [31]. Regarding the effect of DM on the association of e-NOS polymorphisms with clopidogrel resistance in the patients with CAD undergoing PCI, the study was reached

to that only e-NOS polymorphism 4a/4b displayed a marked effect of DM on that association or DM patients at risk factor as (2.4-2.8) more than non-DM patients to develop clopidogrel resistance in CAD patient, therefore our results are consistent with [32] and [33].

In contrast to earlier findings, G894T and T786C variants showed no effect of DM on the association of e-NOS polymorphisms with clopidogrel resistance in patients with CAD undergoing PCI. These findings are similar to the results of [32], [34], [35], and [36] but contradict those of [37].

Furthermore, in our study, the homozygous CC of the T786C variant in the case and control groups had a positive impact of DM on the association of e-NOS polymorphisms with clopidogrel resistance in patients with CAD undergoing PCI, similar to the results of Jeehyeon Bae and his associates, who found reached that e-NOS T786C and 4a/4b polymorphisms are risk factors for DM, especially in CAD patients [33]. Although a previous study in the Korean population performed by Kim et.al found that e-NOS polymorphisms, especially T786C, has a negative impact of DM on the association of e-NOS Polymorphisms with clopidogrel resistance [38].

LIMITATIONS

This study was limited by the absence of a control group for CAD and DM.

CONCLUSION

The target of the present research was to investigate the effect of DM on the association of e-NOS polymorphisms with clopidogrel resistance in patients with CAD undergoing PCI. This study revealed that e-NOS polymorphisms (4a/4b and mutant homozygous of T786C) are risk factors for DM in patients with CAD who are resistant to clopidogrel. Performing the same study using larger and more diverse populations will help confirm the observed associations between e-NOS polymorphisms and clopidogrel resistance and to found an intervention in the improvement of diabetes for those with the mutant e-NOS variants and they are resistant to clopidogrel. The findings of this study have many important implications for future practice.

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